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Dynamic structural changes accompany the production of dihydroxypropanesulfonate by sulfolactaldehyde reductase

Mahima Sharma, Palika Abayakoon, James P Lingford, Ruwan Epa, Alan John, Yi Jin, Ethan D. Goddard-Borger, Gideon J. Davies, and Spencer J. Williams

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3 **Dynamic Structural Changes Accompany the Production of**
4 **Dihydroxypropanesulfonate by Sulfolactaldehyde**
5 **Reductase**
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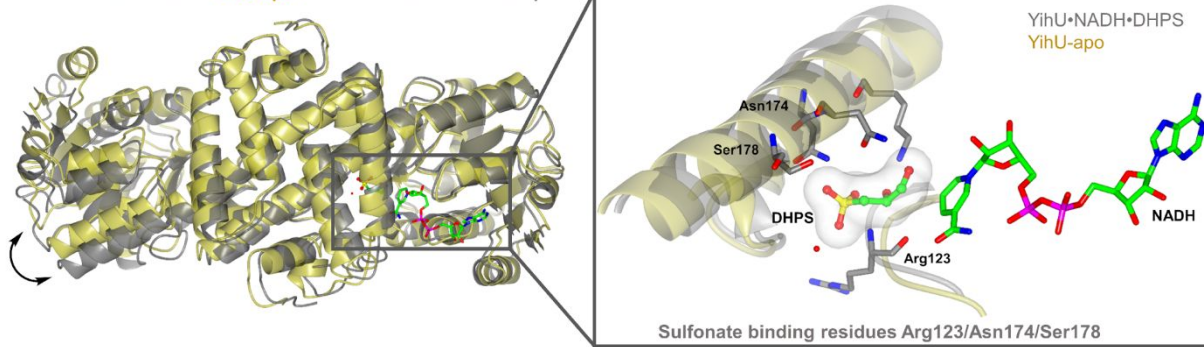
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Abstract

2,3-Dihydroxypropanesulfonate (DHPS) is a major sulfur species in the biosphere. One important route for the production of DHPS is sulfoglycolytic catabolism of sulfoquinovose (SQ) through the Embden-Meyerhof-Parnas (sulfo-EMP) pathway. SQ is a sulfonated carbohydrate present in plant and cyanobacterial sulfolipids (sulfoquinovosyl diacylglyceride and its metabolites) and is biosynthesised globally at a rate of around 10 billion tonnes per annum. The final step in the bacterial sulfo-EMP pathway involves reduction of sulfolactaldehyde (SLA) to DHPS, catalysed by an NADH-dependent SLA reductase. Based on conserved sequence motifs, we assign SLA reductase to the β -hydroxyacid dehydrogenase (β -HAD) family, an example of a β -HAD enzyme that acts on a sulfonic acid substrate, rather than a carboxylic acid. We report crystal structures of the SLA reductase YihU from *E. coli* K-12 in its apo and cofactor-bound states, as well as a ternary complex YihU•NADH•DHPS with the cofactor and product bound in the active site. Conformational flexibility observed in these structures, combined with kinetic studies, confirm a sequential mechanism and provide evidence for dynamic domain movements that occur during catalysis. The ternary complex structure reveals a conserved sulfonate pocket in SLA reductase that recognises the sulfonate oxygens through hydrogen bonding to Asn174, Ser178, and the backbone amide of Arg123, along with an ordered water molecule. This triad of residues distinguishes these enzymes from classical β -HADs that act on carboxylate substrates. A comparison of YihU crystal structures with close structural homologues within the β -HAD family highlights key differences in the overall domain organization and identifies a peptide sequence that is predictive of SLA reductase activity.

Keywords: sulfoglycolysis, X-ray crystallography, alkylsulfonate, NADH-dependent, reductase, three-dimensional structure, bisubstrate enzyme kinetics

Graphical abstract

Domain movements in YihU-*apo* vs. YihU•NADH•DHPS complex

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3 The biodegradation of organosulfur compounds within the biogeochemical sulfur cycle is
4 crucial for recycling this essential macronutrient. 2,3-Dihydroxypropanesulfonate (DHPS) is
5 an important intermediate in the biosulfur cycle and is produced globally on a significant scale
6 from organosulfur precursors by plants, diatoms and bacteria. Bacteria produce DHPS by
7 catabolism of the sulfosugar sulfoquinovose (SQ), which has an estimated annual production
8 of 10^{10} tonnes, through the sulfoglycolysis pathway (Figure 1).¹⁻² DHPS is also a major species
9 in sulfur fluxes through the marine web. Oceanic diatoms produce massive amounts of DHPS,
10 presumably by deamination of cysteinolic acid,³⁻⁵ with production levels on par with the major
11 marine organosulfur species dimethylsulfoniopropionate^{6,7} and dimethylsulfoxonium
12 propionate.⁸ DHPS is the substrate for a range of bacterial biomineralization processes that
13 cleave the carbon-sulfur bond to liberate inorganic sulfite,³ sulfate⁹ or sulfide,¹⁰ or that lead to
14 assorted secondary metabolites.¹¹ For instance, *Desulfovibrio sp.* strain DF1 from anaerobic
15 sewage sludge converts DHPS to hydrogen sulfide,¹⁰ while *Roseobacter* in marine
16 environments convert DHPS to bisulfite.^{12,13} To facilitate these processes, enzymes have
17 evolved to catalyse the inversion of *R*-DHPS, which may represent the stereoisomer formed
18 by deamination of cysteinolic acid, to *S*-DHPS.¹² On the whole, the enzymes and pathways
19 involved in the synthesis and degradation of this significant sulfur-containing metabolite have
20 not been well studied.
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33 The molecular cloning of the sulfoglycolytic Embden-Meyerhof-Parnas (sulfo-EMP)
34 pathway responsible for catabolism of SQ in *E. coli* revealed that the final chemical step
35 involved reduction of the C₃-sulfonate sulfolactaldehyde (SLA) to DHPS by the NADH-
36 dependent reductase YihU, followed by export from the cell.¹ YihU belongs to the β -
37 hydroxyacid dehydrogenase family (β -HADs), a group of enzymes that until the discovery of
38 the SLA reductase activity of YihU were believed to act exclusively on 3-hydroxy carboxylic
39 acid substrates, such as glycerate, 6-phosphogluconate, serine, D-phenylserine, 2-
40 (hydroxymethyl)glutarate and succinate semialdehyde (an earlier report anticipated this result
41 by noting the YihU catalysed NADH-dependent oxidation of 3-hydroxypropanesulfonate).¹⁴ β -
42 HADs feature highly conserved structural folds and conserved sequence motifs for cofactor
43 and substrate binding.¹⁵ β -HADs bind their substrates in a cleft formed between their two
44 domains: an N-terminal Rossmann domain and an all-helical C-terminal dimerization domain.
45 Recently, the 3D structure of a NADPH-dependent dehydrogenase (IsfD) that plays a role in
46 nitrogen assimilation from taurine (aminoethyl sulfonate) was reported.¹⁶ IsfD belongs to the
47 short-chain dehydrogenase/reductase family and catalyses the reduction of sulfoacetaldehyde
48 (SA) to isethionate: this enzyme utilizes a Tyr-Arg-Gln motif to recognize the sulfonate group
49 of these C₂-organosulfonates. IsfD differs from β -HADs in its two-domain structural framework
50 and contains an N-terminal Rossmann fold and a small C-terminal tail formed of two β -strands.
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3 Another group of NADH-dependent SA reductases have been identified from *Bilophila*
4 *wadsworthia* (SarD)¹⁷ and *Bifidobacterium kashiwanohense* (TauF)¹⁸ that belong to the metal-
5 dependent alcohol dehydrogenase superfamily.
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9 In this work, we present a combined sequence, kinetic and structural study of SLA
10 reductase (YihU) from *E. coli* str. K-12. Sequence analysis reveals YihU to be the first example
11 of a β -HAD that acts on a sulfonic acid substrate and identifies a sequence motif conserved
12 among SLA reductases. A biochemical assay established to assess the kinetic properties of
13 YihU confirms that it is a dedicated SLA reductase, with no detectable activity on the
14 analogous glycolytic intermediate glyceraldehyde-3-phosphate. We show that modified NADH
15 analogues are inhibitors of YihU, and that the enzyme acts through a rapid equilibrium
16 sequential mechanism. Finally, we present a series of 3-D X-ray structures of YihU in its apo
17 form, in a binary complex with NADH, and in a ternary product-like complex,
18 YihU•NADH•DHPS. The 3-D structures provide a structural basis for cofactor binding and
19 sulfonate recognition and illuminate the dynamic structural changes that occur during
20 catalysis.
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30 Results and Discussion

31 **Sequence alignment reveals YihU belongs to the β -HAD family.** Alignment of the *E. coli*
32 YihU sequence with that of characterized β -hydroxyacid dehydrogenases (β -HADs) reveals
33 that it shares the four defining motifs that are collectively involved in cofactor-binding,
34 substrate-binding and catalysis (Figure 2A).¹⁵ Motif-1 of β -HADs consists of
35 GXXGXGXMGXXXAXNXXXXG and contains the dinucleotide cofactor binding residues;
36 motif-2 consists of substrate-binding sequence DAPVSGGXXXAXXG; motif-3 consists of
37 GXXGXGXXXKXXXN/Q, which contains the active site lysine and conserved Asn/Gln residue;
38 and motif-4 derives from the C-terminal domain comprising KDLGXAXD sequence and shows
39 a high degree of conservation among bacterial homologues. Examination of the sequence
40 alignment of a phylogenetically-related putative SLA reductases (assigned based on context
41 within gene clusters encoding the sulfo-EMP pathway) from a selection of alpha-, beta- and
42 gammaproteobacteria along with other β -HADs including 3-hydroxyisobutyrate
43 dehydrogenases, 2-hydroxymethylglutarate dehydrogenases, 2-hydroxyl-3-oxopropionate
44 reductases, and tartronate semialdehyde reductases, shows that SLA reductases form a
45 distinct sub-group within the β -HAD family. The sequence alignment reveals that the key
46 differences in the YihU sequences lie within motif-2 where Gly122-Arg123-Thr124 replace the
47 conserved Ser-Gly-Gly seen in β -HADs (Figure 2B). Based on these sequence alignments
48 that reveal the putative SLA reductases form a distinct subgroup, we define an extended motif-
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3 2 [D/EVPVGR~~XXX~~AXXG] as a 'sulfonate substrate-binding motif' common to all SLA
4 reductases.
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8 **YihU SLA reductase does not reduce GAP and follows a rapid equilibrium sequential**
9 **kinetic mechanism.** Reaction rates for YihU catalysed conversion of racemic D/L-SLA¹⁹ to
10 DHPS were measured by monitoring absorbance at 340 nm for enzymatic NADH oxidation to
11 NAD⁺. By varying the concentration of D/L-SLA and keeping NADH constant (0.10 mM) or *vice*
12 *versa* using constant D/L-SLA (5.00 mM), we could perform Michaelis-Menten kinetic analyses
13 for the two substrates. Kinetic parameters were calculated for the concentration of D-SLA,
14 assuming that L-SLA was not a substrate. Both NADH and SLA exhibited saturation kinetics,
15 allowing calculation of k_{cat} , $K_{\text{M}}^{\text{app}}$ and $k_{\text{cat}}/K_{\text{M}}^{\text{app}}$ values under pseudo first order conditions
16 (Figure 3A,B). At [NADH] = 0.1 mM, under conditions of varying [SLA] we determined $K_{\text{M}}^{\text{app}}$ =
17 0.3 mM, k_{cat} = $3.3 \times 10^2 \text{ s}^{-1}$, and $k_{\text{cat}}/K_{\text{M}}^{\text{app}}$ = $1.09 \times 10^3 \text{ mM}^{-1} \text{ s}^{-1}$. At [D-SLA] = 2.5 mM, under
18 conditions of varying [NADH] we determined $K_{\text{M}}^{\text{app}}$ = 0.082 mM, k_{cat} = $5.48 \times 10^2 \text{ s}^{-1}$, and
19 $k_{\text{cat}}/K_{\text{M}}^{\text{app}}$ = $6.72 \times 10^3 \text{ mM}^{-1} \text{ s}^{-1}$ (for full data and associated errors see Table 1). No activity
20 was observed for reduction of racemic glyceraldehyde phosphate (GAP)²⁰ under similar
21 conditions. GAP is produced from DHAP through the action of triose phosphate isomerase,
22 and in the first step of lower glycolysis undergoes conversion to 1,3-bisphosphoglycerate by
23 the action of GAP dehydrogenase (GADPH). The lack of activity of YihU on GAP prevents
24 interference with this important glycolytic/gluconeogenic intermediate. Saito *et al.* reported that
25 YihU catalyzes succinate semialdehyde reduction with kinetic parameters of $V_{\text{max}} = 0.20 \pm 0.04$
26 $\text{mmol min}^{-1} \text{ mg}^{-1}$ and $K_{\text{M}}^{\text{app}} = 4.3 \pm 1.2 \text{ mM}$ (at 1 mM NADH),¹⁴ which we calculate equates to
27 $k_{\text{cat}}/K_{\text{M}}^{\text{app}} = 26 \pm 12 \text{ M}^{-1} \text{ s}^{-1}$. Thus, YihU exhibits a 42,000-fold greater catalytic efficiency for the
28 reduction of SLA over SSA. Collectively, these data confirm that YihU is a dedicated SLA
29 reductase.
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44 Multi-substrate enzymes perform catalysis through two main mechanisms: the
45 sequential (ternary complex) mechanism, in which both substrates must bind before a
46 chemical step leading to product formation; or the ping-pong mechanism, in which one or more
47 products are released prior to binding of all substrates. The Theorell-Chance mechanism is a
48 special case in which there is a defined order of substrate association and product release
49 without accumulation of a ternary complex. As SLA reductase has two substrates and two
50 products, its molecularity is described as Bi-Bi. For β -HADs²¹ and other dehydrogenases,^{22,23}
51 a sequential Bi-Bi mechanism is often reported with the binding of the redox-active cofactor
52 preceding localisation and binding of the substrate.
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3 For a bisubstrate enzyme with substrates A and B, plotting $1/v_0$ versus $1/[A]$ at various
4 constant concentrations of substrate B or $1/v_0$ versus $1/[B]$ at various constant concentrations
5 of substrate A can indicate the mechanism of the reaction.²⁴ For a ping-pong mechanism,
6 plotting $1/v_0$ versus $1/[A]$ affords a series of straight lines with slope of $K_M(A)/V_{max}$. In contrast,
7 for a classical sequential mechanism plotting $1/v_0$ versus $1/[A]$ will produce a family of straight
8 lines with slope depending on the concentration of B that intersect to the left of the y -axis, or
9 in the case of a rapid equilibrium sequential mechanism, on the y -axis.²⁵ To study the kinetic
10 mechanism used by YihU we simultaneously varied the concentration of SLA while NADH was
11 held at saturation ($[NADH] = 0.05-0.30$ mM) and *vice versa* ($[D/L-SLA] = 2.50-12.0$ mM), at a
12 constant concentration of YihU. The resulting double-reciprocal plots yielded patterns of lines
13 that intersected on the y -axis (Figure 3C,D). For the plot of $1/[NADH]$ (at different SLA
14 concentrations) the data intersected the y -axis above zero; for the plot of $1/[SLA]$ (at different
15 NADH concentrations) the data clearly intersects on the origin. These patterns directly rule
16 out a ping-pong mechanism for YihU. While intersection on the y -axis is unusual, it has been
17 reported for creatine kinase by Schimerlik and Cleland, who derived the corresponding kinetic
18 equations, and showed that the data could indicate which substrate bound first.²⁵ In line with
19 the analysis of Schimerlik and Cleland, the patterns observed here are consistent with a rapid
20 equilibrium sequential mechanism, and indicate initial binding of NADH. The kinetic
21 mechanism allows proposal of a catalytic mechanism for SLA reductases that is consistent
22 with that proposed for other β -HADs and involves ordered binding of the two substrates NADH
23 and SLA to form a ternary complex, followed by a chemical step involving hydride transfer
24 from NADH and protonation of the substrate.
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40 We synthesized two NADH analogues by partial (tetrahydro-NADH) and complete
41 reduction (hexahydro-NADH) of the nicotinamide ring of NADH, as described by Dave *et al.*²⁶
42 and assessed these compounds as inhibitors of YihU. Owing to the small amounts of these
43 compounds available we limited our studies to determination of IC_{50} values. Under conditions
44 of $[SLA] = K_M(SLA)/10$ and $[NADH] = K_M(NADH)$ we measured IC_{50} values of 4.03 and 10.3
45 mM, respectively (Figure S1). These data reveal that tetrahydro-NADH is a better inhibitor
46 than hexahydro-NADH, as might be expected considering its greater structural resemblance
47 to the cofactor. Disappointingly, we were unable to obtain X-ray structures of these inhibitors
48 bound to YihU.
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55 **YihU forms a dimer of intimate homodimer pairs.** In order to identify the amino acid
56 residues involved in substrate binding and catalysis, we solved the X-ray structure of YihU.
57 Despite conserved sequence motifs and moderate sequence similarity with other β -HADs, we
58 could not achieve a structure solution using a single model. The YihU structure was solved
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3 using the molecular replacement pipeline BALBES²⁷ that selected a human
4 hydroxyisobutyrate dehydrogenase as the reference structure (PDB ID 2GF2 with 31%
5 sequence similarity). Data collection and refinement statistics for YihU structures are given in
6 Table S1. YihU crystallised as a dimer of dimers with four molecules present in the asymmetric
7 unit (Figure 4A). Size exclusion chromatography-multiangle light scattering (SEC-MALS)
8 confirmed that YihU also exists as tetramer in solution (Figure S2). Within the asymmetric unit,
9 each protomer adopts a two-domain architecture containing a N-terminal nucleotide binding
10 domain (residues 1-164) and a C-terminal helical bundle (residues 165-294) both connected
11 by long inter-domain helix $\alpha 8$ (Figure S3). The N-terminal domain is composed of a classical
12 α/β Rossmann fold (comprised of an extended sheet formed of $\beta 1-6$, flanked by $\alpha 1-5$ in a
13 three-layered sandwich) appended with an additional $\beta-\alpha-\beta$ motif containing $\beta 7-9$ and $\alpha 6-7$.
14 An intimate homodimer pair between two monomers is formed through 3D domain swapping
15 of the dimerization domains involving C-terminal helices $\alpha 8-14$. This dimerization domain is
16 formed when the central trans-domain helix $\alpha 8$ from one monomer (A) inserts into the C-
17 terminal helical bundle from the opposite monomer (B) making several reciprocal interactions
18 through both charged and hydrophobic residues (Figure S4).

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30 **The tetrameric assembly and domain organization of YihU matches that of imine**
31 **reductases (IREDs).** Four interfaces are present within the subunits of the YihU tetramer: A-
32 B (Interfaces I and II), A-C (Interface III) and A-D (Interface IV) (Figure 4A, Figure S5). PISA
33 analysis of the AB dimer assembly indicated a total buried surface area of 10,638 Å². The
34 interface area for chains A-B is 4,023 Å², which corresponds to 38% of the total, indicating an
35 intimate homodimer pair. At the major interface, interface I, the C-terminal bundle of subunit
36 A (shown in grey) interacts with C-terminal domain of subunit B to form a hydrophobic helical
37 core. Interface II also occurs in the AB dimer and harbours the active site formed by reciprocal
38 domain sharing between C-terminal helices (A) and the N-terminal Rossmann domain of chain
39 B, indicating that dimer assembly is essential for catalytic activity of YihU. Conserved residue
40 Lys171 in motif-3 projects into an inter-domain cleft that is lined mainly by charged, polar
41 residues (Table S2). Interfaces III and IV comprise minor interfaces and are formed by
42 hydrogen bonding interactions between subunits A-C and A-D, respectively. These interfaces
43 appear to be important for assembly of the tetramer and may contribute to the overall stability
44 of the enzyme.
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55 A DALI search using YihU against the RCSB PDB library revealed that its closest
56 structural neighbours belong to the β -HAD family. The closest structural homologues included
57 human 3-hydroxyisobutyrate dehydrogenase (PDB ID: 2GF2 with DALI z score of
58 31.6, rmsd 2.2 and 31% sequence ID), tartronate semialdehyde reductase from *Salmonella*
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3 *typhimurium* LT2 (1VPD²⁸ with DALI z score of 31.5 and 32% sequence ID) and 6-
4 phosphogluconate dehydrogenase from *Pyrobaculum calidifontis* (3W6U²⁹ with z score of 31.4
5 and 31% sequence similarity). Several other annotated hydroxyisobutyrate dehydrogenases
6 (HIBDHs) that have been reported to reduce imine substrates (imine reductases or IREDs)
7 were also identified including 5OCM,³⁰ 5G6S,³¹ and 6EOD³² with low sequence identity (16-
8 20%) but relatively high DALI z scores of 25-27 and overall rmsd of 2.2-3.2, reflecting high
9 structural similarities. Superposition of monomers of human HIBDH (2GF2) and the IRED from
10 *Streptosporangium roseum* (5OCM) with YihU shows all three proteins contain a common
11 two-domain structure comprising N-terminal Rossmann-like fold and C-terminal helical bundle
12 connected by a long transdomain helix. However, while superposition of the isolated
13 Rossmann domain and C-terminal helices of YihU monomer over the two domains of human
14 HIBDH shows high fold conservation (RMSD 1.14Å/160 residues and 1.32Å/74 residues for
15 N- and C-terminal domains, respectively), the quaternary organization of the subunits display
16 large differences. In human HIBDH, α 9 helix of C-terminal domain takes a sharp turn to
17 interact with N-terminal domain from the same monomer. As a result, the two monomers in
18 human HIBDH sit adjacent to one another and only interact along the length of long α 8 helix,
19 arranged in a back-to-back fashion in a dimer pair (Figure 4B). On the other hand, the two
20 domains in YihU are rewired such that their subunit organization is similar to IREDs.³³ YihU
21 and IREDs possess homodimeric folds formed by extensive domain swapping. In YihU (or the
22 equivalent helix in IREDs), helix α 9 of monomer A adopts an extended conformation so that
23 the C-terminal helices (of chain A) travel further away and form an active site cleft with the N-
24 terminal domain of opposite monomer B (Figure 4C, Figure S6).

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40 **A binary YihU•NADH complex reveals dynamic domain movement upon cofactor**
41 **binding.** Co-crystallization of YihU with NADH afforded a binary complex showing clear
42 density for the cofactor bound to the N-terminal Rossmann domain of all four monomers within
43 the asymmetric unit (Figure 5A-B, Figure S7). Domain conformational motion analyses of the
44 apo and YihU•NADH structures, using the DynDom program,³⁴ reveals the former adopts an
45 'open' and the latter a 'closed' conformation, as a result of two dynamic domains with a
46 bending region comprised of residues 156-166 connecting the two domains. Cofactor binding
47 results in an 8° inter-domain rotation to form a more compact active-site pocket that
48 encapsulates the NADH molecule. The NADH molecule binds in a *syn* conformation and the
49 2'-hydroxyl of ribose ring is hydrogen-bonded to Asp31, a residue that provides specificity for
50 NADH. By contrast, NADPH binding β -HAD homologues possess Asn at this position, usually
51 followed by an arginine residue that makes stacking interactions with adenine and binds to 2'-
52 phosphate of NADPH. The structure shows that NADH specificity likely arises from
53 destabilizing interactions of Asp31 that would occur with the 2'-phosphate in NADPH. The
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3 nicotinamide ring of NADH projects into a relatively narrow cleft formed as a result of domain
4 closure upon binding of the cofactor.
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8 **YihU possesses a defined substrate channel for entry of SLA to the active site.** Two
9 pores that could possibly provide for entry of SLA into the active site (as defined by the location
10 of the catalytic Lys171) were identified in the YihU-apo structure. However, upon binding of
11 NADH to form the YihU•NADH complex, domain movement leads to closure of the active site,
12 blocking one of these pores. At the entry to the other pore, Arg123 (chain A) and Lys213 (chain
13 B) contribute to a positively charged surface patch which may facilitate entry of the negatively
14 charged substrate SLA (Figure 5C).
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21 Using CAVER Web 1.0,³⁵ a tool for visualization and analysis of tunnels and channels
22 in protein structures, we selected Lys171 in the YihU•NADH structure as the reference starting
23 point. A 10.4 Å long tunnel was visualised, which could provide a pathway for entry of the
24 substrate SLA (and possibly release of the product DHPS). This tunnel tapers to a bottleneck
25 at the back of the sulfonate-binding pocket to confine the dimensions of the substrate pocket
26 (Figures S8-9). Thus, the residues forming the sulfonate binding pocket, the residues at the
27 entry to this predicted tunnel, and its size may contribute to the substrate specificity of YihU,
28 possibly involving electrostatic filtering of anionic substrates at the entrance to the tunnel
29 (Table S2).
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36 **A ternary YihU•NADH•DHPS complex reveals the sulfonate binding pocket and active**
37 **site residues.** A ternary complex was obtained with NADH and the product DHPS, by soaking
38 solid racemic DHPS directly onto a YihU•NADH crystal in mother liquor. Close examination of
39 the 3D structure revealed the two dimer pairs (comprised of four chains in the asymmetric unit)
40 are present in different conformations with varying ligand densities observed close to
41 Ser178/Asn174 in the active site cleft. The NADH cofactor was found to be somewhat mobile
42 in the different chains in the structure upon soaking DHPS at high concentrations, resulting in
43 apo conformations in two chains and a binary YihU•DHPS complex in one of the chains. In
44 one monomer, clear density was seen for both the NADH cofactor and another ligand bound
45 at the active site that allowed modelling of the natural isomer S-DHPS (Figure 6A). S-DHPS
46 sits within the active site and is anchored to Lys171 by hydrogen-bonds to C-1 (2.5 Å) and
47 C-2 (2.9 Å) hydroxyls, and flanked by bulky, hydrophobic residues on the other side (Phe233
48 and Trp279 from the opposite subunit B). A well-ordered binding pocket surrounds the
49 sulfonate group of S-DHPS. One sulfonate oxygen makes hydrogen bonds with Ser178 and
50 Asn174 at 2.6 and 3.1 Å, respectively; the second oxygen is hydrogen-bonded to Asn174 (2.9
51 Å) and the backbone amide N-H of Arg123 (2.9 Å); and the third sulfonate oxygen makes a
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3 hydrogen bond with the bound water molecule (Figure 6B). This water molecule is 3 Å from
4 the backbone amide of Ala210 (not a conserved residue) and at 2.7 Å and 3.3 Å distance from
5 two ordered water molecules; one of these H-bonds to side-chain hydroxyl of Ser219.
6 Crucially, this sulfonate binding triad of residues Arg123-Asn174-Ser178 is conserved in all
7 assigned bacterial SLA reductases (Figure 2A).
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12 While S-DHPS is the product of the reaction, within the YihU•NADH•DHPS complex
13 NADH and S-DHPS are positioned in what can be considered a catalytically relevant
14 conformation, with C-1 of DHPS situated 3.3 Å away from C4 of nicotinamide ring. As D-SLA
15 would make many of the same interactions as S-DHPS and NADH is seen bound in a *syn*
16 conformation in our ternary complex, this confirms that reduction of SLA by NADH will involve
17 transfer of hydride from the *si*-face of NADH (Figure 6C).
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23 **Identification of the structural basis for binding of sulfonate and carboxylate substrates**
24 **in the β-HAD family.** In order to understand how sulfonate/carboxylate selectivity arises within
25 the β-HAD family, we compared the structures of the YihU•NADH•DHPS complex and that of
26 *Salmonella typhimurium* GarR-tartronate semialdehyde reductase bound to a substrate
27 analog, L-tartrate.²⁸ A key difference is visible within motif-2, which contains the substrate
28 binding loop that interacts with the carboxylate/sulfonate groups of the ligands (Figure 7). In
29 YihU, Arg123 within the triplet Gly122-Arg123-Thr124 interacts with one sulfonate oxygen,
30 whereas in GarR the equivalent (and conserved among classical carboxylate β-HADs)
31 Ser123-Gly124-Gly125 residues exhibit a 180° flip in the central glycine (possibly enabled by
32 the conformationally flexible C α backbone of the two glycines) allowing one carboxylate
33 oxygen to bind the backbone amides of Gly124 and Gly125, present at 2.7 Å and 2.9 Å
34 respectively, and the other carboxylate oxygen to hydrogen-bond to side-chain hydroxyl of
35 Ser123 (2.6 Å).
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46 We used site-directed mutagenesis to explore the role of the sulfonate binding motif in the
47 function of YihU. Amino acids in the triplet Gly122-Arg123-Thr124 were individually converted
48 to the corresponding residue in GarR and Michaelis-Menten parameters of the resulting
49 variant proteins were measured under pseudo-first order conditions (Figure S11). All mutant
50 enzymes suffered a reduction of catalytic efficiency (k_{cat}/K_M^{app}). The change in k_{cat}/K_M^{app} for
51 NADH at constant [SLA] for each of the mutants versus the wildtype was small, in the range
52 of 3-12-fold reduction, consistent with the sites of mutation involving interaction with SLA
53 (Table 1). Conversely, the change in k_{cat}/K_M^{app} for SLA at constant [NADH] for each of the
54 mutants relative to wildtype was greater, comprising a 25-, 130- and 230-fold reduction for the
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3 G122S, R123G and T124G mutants, respectively. For the three mutants, K_M^{app} for SLA at
4 constant [NADH] was increased versus wildtype.
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8 **Conclusion**

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10 As the final step in the sulfo-EMP pathway, the NADH-dependent SLA reductase YihU is an
11 important enzyme in sulfur cycling in the biosphere. Our data reveals that YihU acts
12 specifically on SLA, supporting an exclusive role in the *E. coli* sulfo-EMP pathway in reduction
13 of SLA to the excreted metabolite DHPS. Absence of activity on GAP shows that YihU is
14 unlikely to interfere with lower glycolysis or gluconeogenesis. A previous study by Saito *et al.*
15 using recombinant *E. coli* YihU revealed that this enzyme possessed succinate semialdehyde
16 reductase activity,¹⁴ however, our data shows that it possesses a 42,000-fold preference for
17 SLA in terms of k_{cat}/K_M . Indeed, since the sulfo-EMP gene cluster is overexpressed when *E.*
18 *coli* is grown on SQ,¹ reduction of SSA is not only catalytically insignificant but may also be
19 physiologically unimportant. Interestingly, Saito *et al.* demonstrated that 3-
20 hydroxypropanesulfonate could be oxidized by YihU in the presence of NAD⁺.¹⁴ While 3-
21 hydroxypropanesulfonate is not a naturally occurring metabolite, it is the 2-deoxy analogue of
22 DHPS and so this observation foreshadowed the subsequent discovery of the role of YihU in
23 sulfoglycolysis. Our kinetic data demonstrates a rapid equilibrium sequential mechanism. The
24 acquisition of YihU•NADH binary and YihU•NADH•DHPS ternary crystal complexes are
25 consistent with a sequential mechanism involving initial binding of NADH. Binding of a
26 sulfonate substrate occurs through a triad of conserved residues (Arg123-Asn174-Ser178)
27 common to all putative SLA reductases. It is only known in one other case how enzymes in
28 the sulfo-EMP pathway recognize a sulfonate group: 3-D structures of sulfoquinovosidases
29 that catalyze hydrolysis of SQ glycosides revealed a highly conserved Tyr-Arg-Trp triad.^{36,37}
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43 YihU possesses the four characteristic motifs that define members of the β -HAD
44 family,¹⁵ and represents the first β -HAD that acts on a β -hydroxysulfonic acid, rather than a β -
45 hydroxycarboxylic acid. We identified an SLA-specific sequence *DVPVGR*T within motif-2 that
46 distinguished putative SLA reductases from classical β -HADs. In the YihU•NADH•DHPS
47 complex, a hydrogen bond between one of the sulfonate oxygens and the backbone N-H of
48 Arg123 (within the triplet Gly122-Arg123-Thr124) appears particularly important. Conversely,
49 in the case of tartronate reductase GarR, recognition of a carboxylate substrate is achieved
50 by backbone chain flipping in the equivalent sequence triplet Ser123-Gly124-Gly125, resulting
51 in a fundamentally different binding mode of the carboxylate involving side-chain hydrogen
52 bonding with Ser123 and backbone N-H interactions with Gly124 and Gly125. Kinetic analysis
53 of mutant enzymes created by converting each residue with the Gly122-Arg123-Thr124 triplet
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3 of YihU to the corresponding residues in the Ser123-Gly124-Gly125 triplet of GarR are
4 consistent with the roles of these residues in binding the sulfonate of SLA.
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8 SLA reductases possess a close sequence relationship with hydroxyisobutyrate
9 dehydrogenases (HIBDHs) within the β -HAD family. However, analysis of the domain
10 arrangement seen in 3D X-ray structures highlights an alternative wiring pattern and a closer
11 structural resemblance to imine reductases (IREDs), a sub-group of NADPH-dependent
12 dehydrogenases that can reduce imines to amines. As seen in IREDs,³⁰⁻³³ extensive swapping
13 of C-terminal helical bundles between the YihU protomers results in an active site formed at
14 the inter-domain cleft of a dimer pair. Examination of monomeric (1VPD, a model β -HAD) and
15 quaternary domain-swapped (YihU) conformations reveals that while consensus sequence
16 motifs and the overall monomer topology of β -HADs are retained, the substrate-binding motif-
17 2 and catalytic motif-3 from opposite monomer partners in the YihU dimer pair make H-
18 bonding interactions with the sulfonate and facilitate the binding of the natural substrate.
19 Modelling allowed identification of a tunnel leading to the DHPS-binding subsite, and hint at a
20 possible electrostatic gating mechanism for achieving substrate selectivity by positively
21 charged surface residues (Arg123 and Lys213, also from opposite monomers) present at the
22 tunnel entrance.
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33 Because SQ is a major organosulfur species produced by essentially all photosynthetic
34 organisms, its biomineralization is a major contributor to the global sulfur cycle. As one of just
35 two known pathways for catabolism of SQ (the other consisting of the sulfoglycolytic Entner-
36 Doudoroff pathway that leads to sulfolactate),³⁸ the sulfo-EMP pathway is important for sulfur
37 cycling and for production of DHPS for downstream biomineralization by other members of
38 the bacterial community. The insights provided here into the kinetics, structure and function of
39 SLA reductases deepen our understanding of this important arm of the biogeochemical sulfur
40 cycle and will support future bioinformatic analysis of these enzymes.
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48 **Associated content**

49 The Supporting Information is available free of charge on the ACS Publications website at
50 DOI: 10.1021/acscchembio.xxx.
51

52 Tables S1–S2, Figures S1–S11, supporting methods on chemical synthesis procedures,
53 cloning, protein expression and purification, enzyme kinetics, protein crystallization and
54 X-ray crystallography and structure analysis (PDF)
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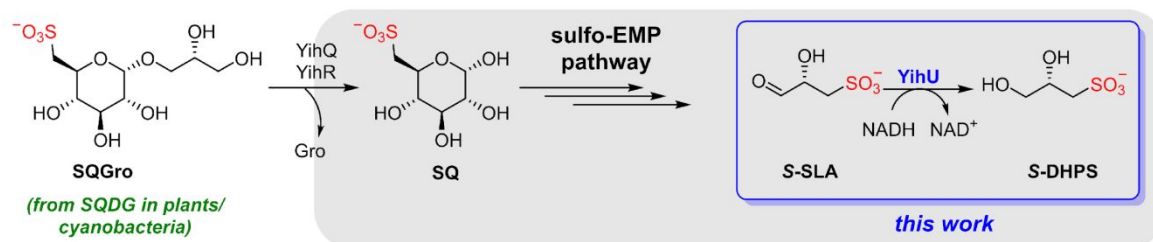
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A. Sulfoglycolysis via DHPS



B. Desulfonation of DHPS

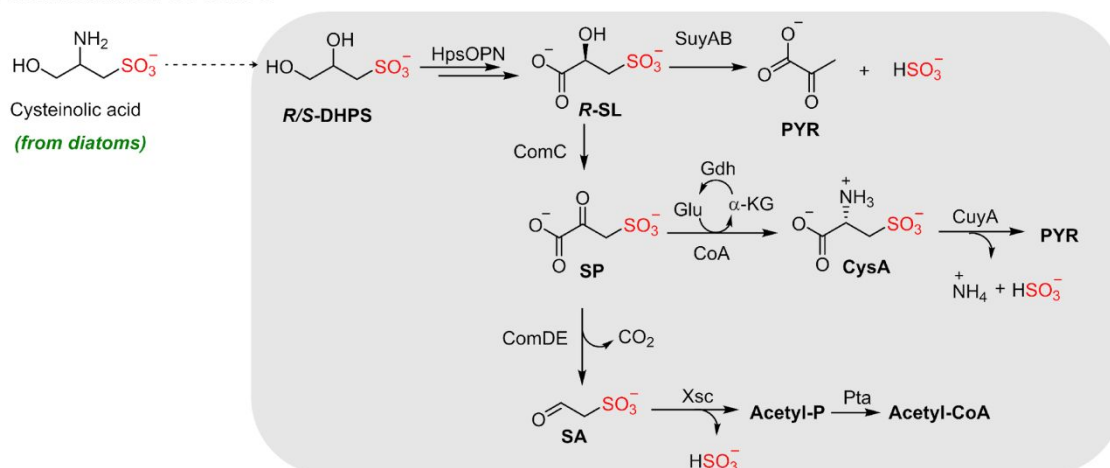


Figure 1. General pathways for cycling of sulfur from organosulfur sources via DHPS. A, Sulfo-EMP pathway in bacteria for degradation of SQDG to give S-DHPS. YihU-catalyzed reduction of SLA to DHPS is shown in blue. B, Degradative pathways for mineralization of sulfur from DHPS. Biosynthesis of DHPS by putative deamination of cysteinolic acid is likely catalysed by CoA/ComC homologues.

A



B

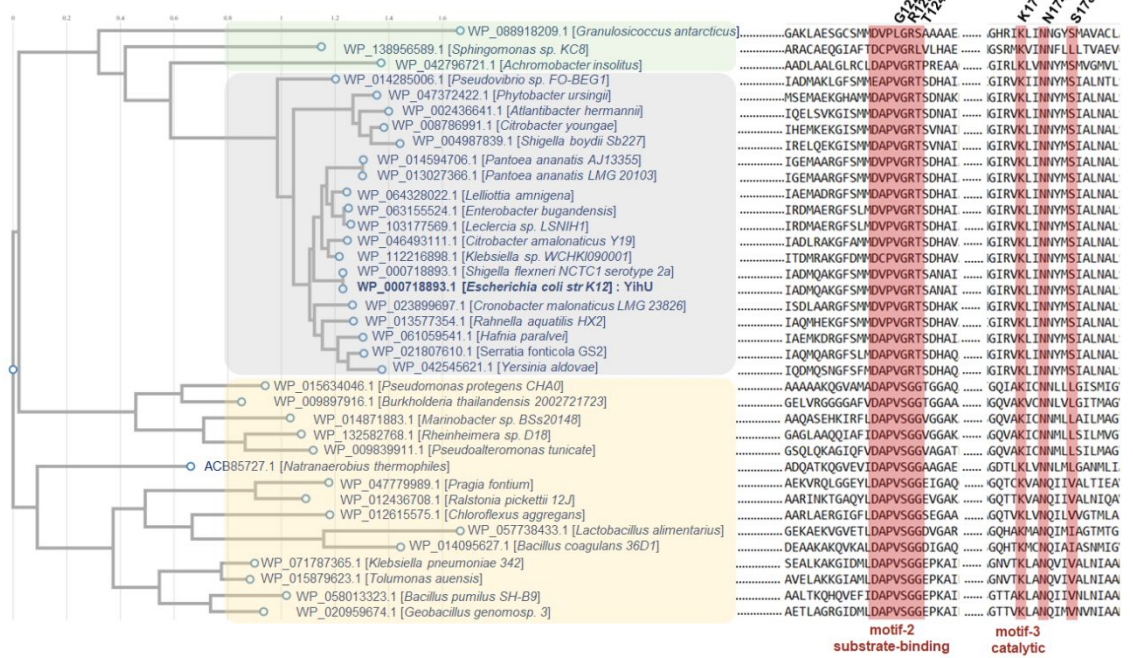


Figure 2. SLA reductases share β -HAD sequence motifs but possess a unique sulfonate substrate binding sequence. A, Sequence alignment of YihU with model β -HADs to show four consensus sequence motifs [motif 1 – cofactor binding GXXGXG sequence; motif 2 – substrate binding; motif 3 containing catalytic lysine; motif 4 – cofactor binding]. All four motifs are also conserved in YihU with the only differences in the substrate binding sequence highlighted in motif-2 residues: G122-R123-T124. B, Phylogenetic tree showing relationship of SLA reductases (grey box) and β -hydroxy acid dehydrogenases (yellow box); consensus β -HAD sequence motifs for substrate binding motif-2 and catalytic motif-3 are indicated. SLA reductases form a separate sub-group with conserved 'sulfonate substrate binding sequence' at residues 122-124 (YihU numbering). Also highlighted is a clade comprised of other annotated β -HADs with predicted roles in sulfur metabolism (green box).

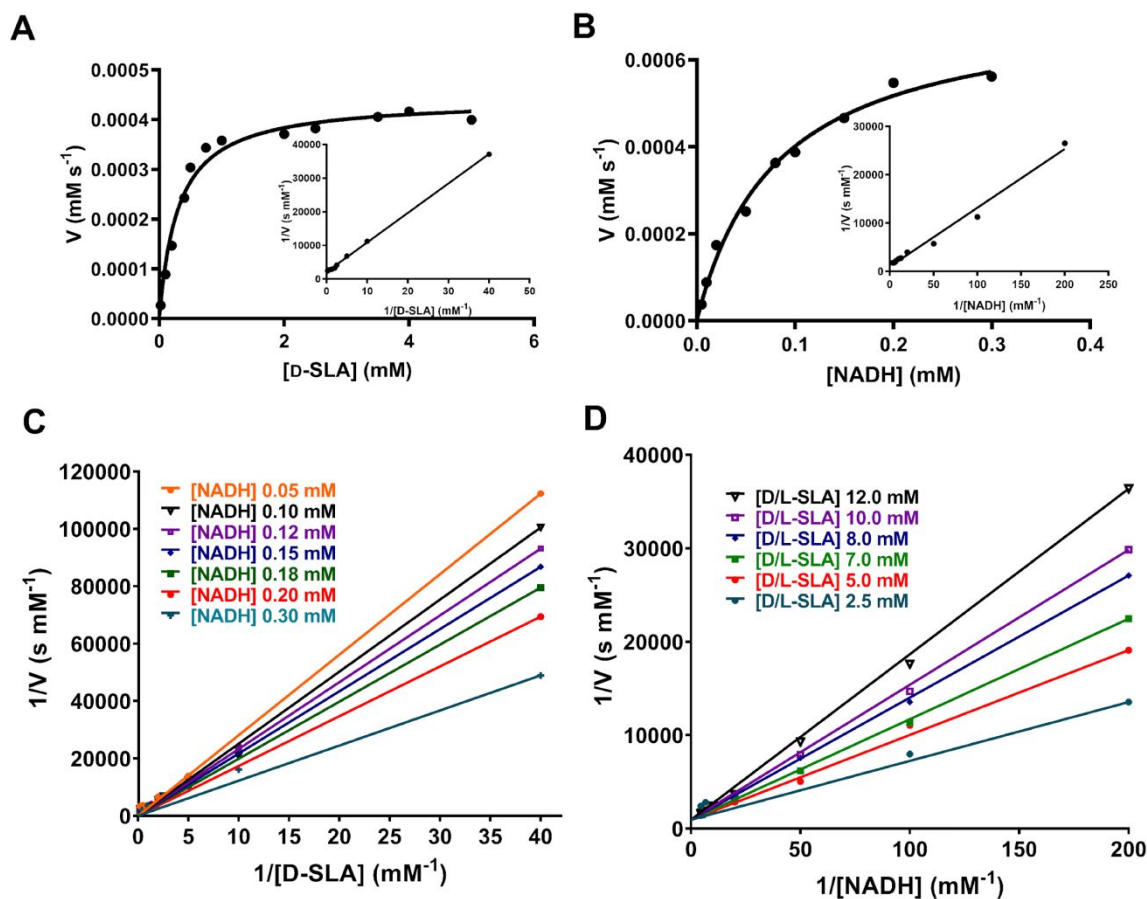


Figure 3. Kinetic studies and double reciprocal plots for rapid equilibrium sequential binding mechanism. A-B, Michaelis-Menten and Lineweaver-Burk (inset) plots for reduction of SLA to DHPS by YihU under pseudo first order conditions of $[NADH] = 0.1$ mM (for A) and $[D-SLA] = 2.5$ mM (for B). C-D, Double reciprocal plots indicate a rapid equilibrium sequential mechanism for YihU. D-SLA concentration was varied at several fixed concentrations of NADH (0.05–0.30 mM). D, NADH concentration was varied at several fixed concentrations of DL-SLA (2.5–12.0 mM).

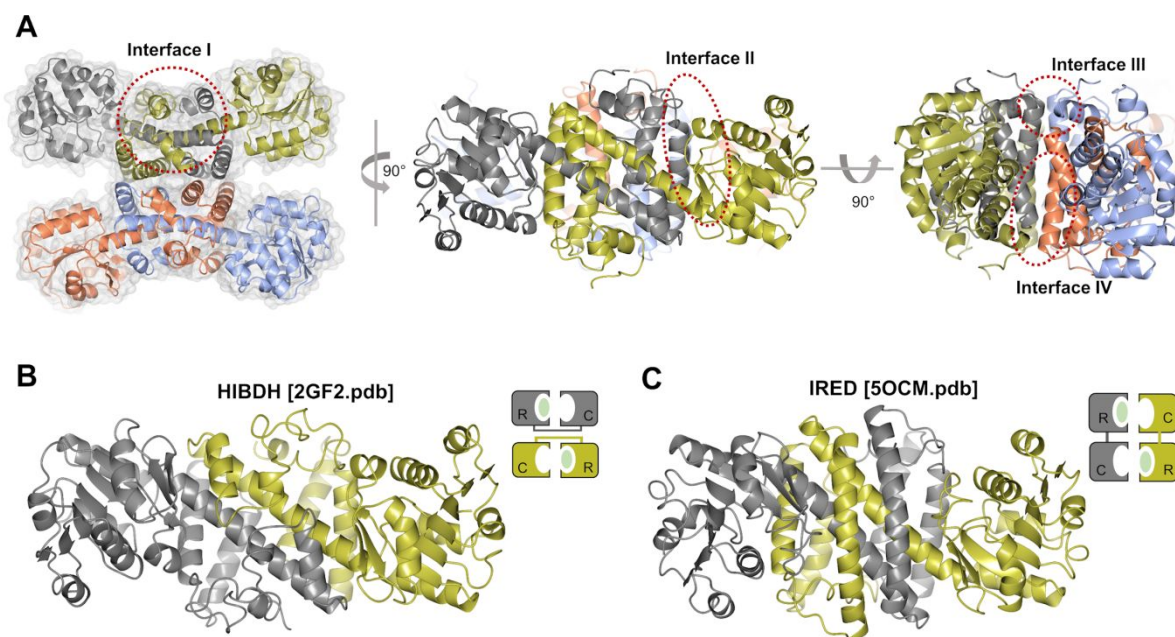


Figure 4. Domain organisation of YihU and comparison with HIBDHs and IREDs. A, Overall structure of YihU-apo depicted as dimer-of-dimers in different orientations (related by 90° rotation) to show interfaces I-IV. B-C, Representative dimer pairs from crystal structures of human HIBDH (2GF2) and the IRED from *Streptosporangium roseum* (5OCM) showing differences in domain organization between HIBDH vs. IRED/YihU enzymes. For each enzyme, the protomers from each dimer pair are depicted in different colours for clarity (in grey and gold or coral and blue).

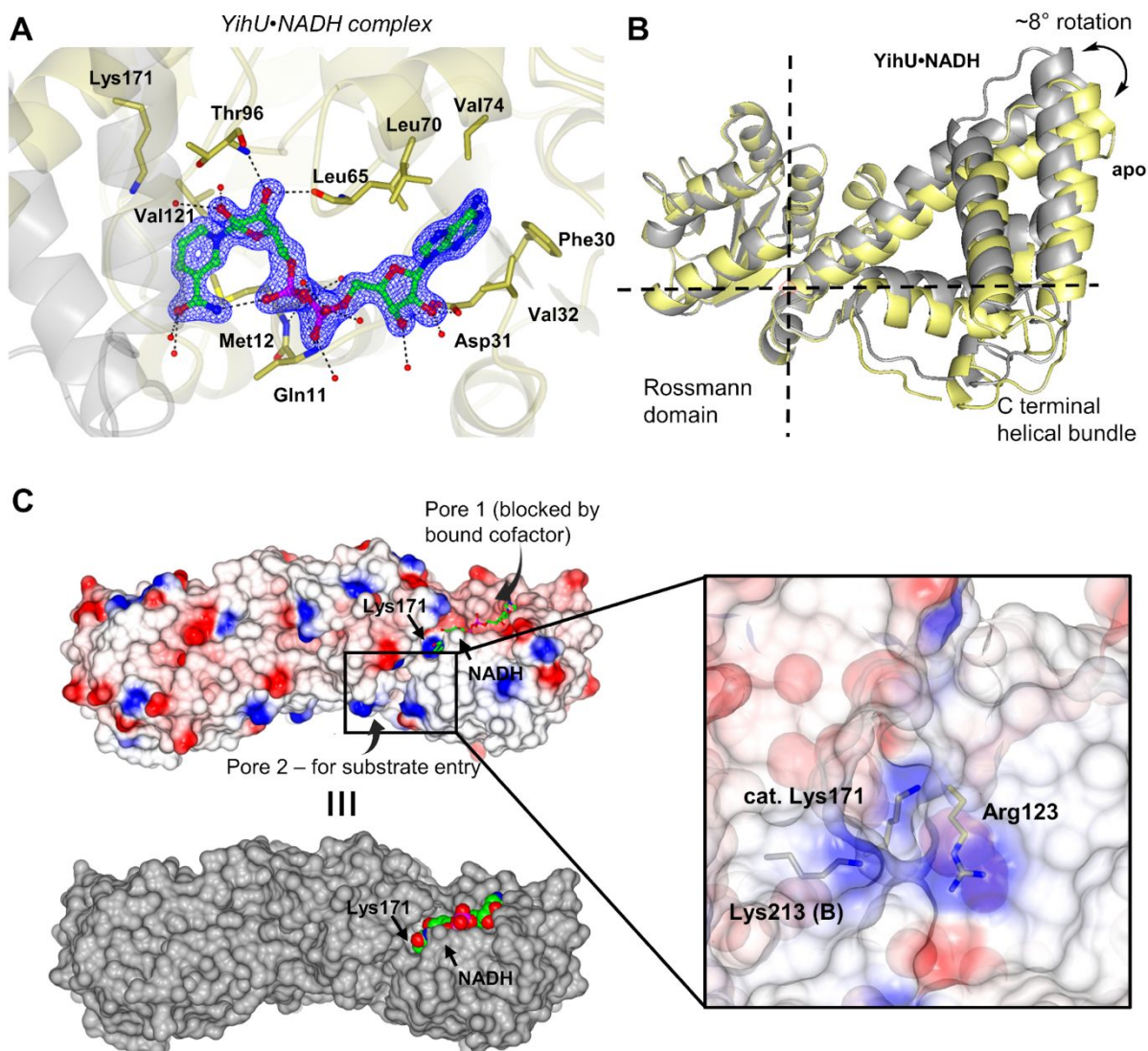


Figure 5. Dynamic domain movements revealed in the complex of YihU•NADH. *A*, Close-up of NADH bound to YihU. Backbone and carbon atoms of subunits A and B are shown in gold and grey, respectively, and NADH and DHPS are shown in cylinder format. Electron density corresponds to the $2F_o - F_c$ and in blue at levels of 1.5σ . *B*, DynDom analysis of the dynamic domains and hinge bending motion of the YihU-apo (depicted in gold) vs. NADH bound conformation (in grey); the lines cross at the centre of rotation and the hinge axis is perpendicular to this crossing point. *C*, Electrostatic potential depiction of YihU-apo dimer surface showing positively charged patch on the surface gated by Arg123 and Lys213. The 'pore' was visualized from surface to catalytic Lys171 in the active site (see Figure S9).

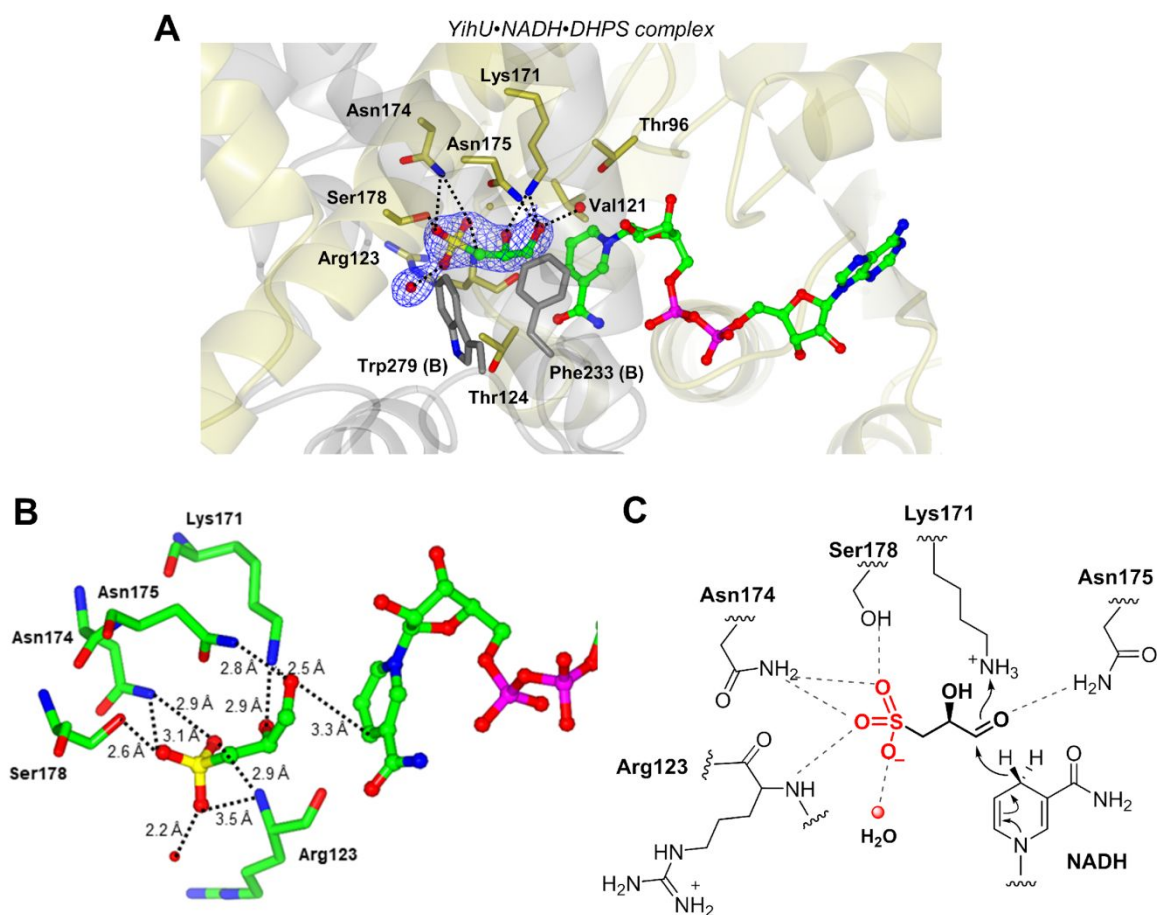


Figure 6. Active site and mechanism of SLA reductase YihU. A, Ternary complex structure of YihU with NADH and DHPS bound at the active site showing the sulfonate pocket. Backbone and carbon atoms of subunits A and B are shown in gold and grey, respectively, and NADH and DHPS are shown in cylinder format. Electron density corresponds to the $2F_o - F_c$ map (in blue) at levels of 1.2σ . B, Detailed view of substrate binding pocket of YihU as observed in the ternary complex depicting hydrogen bonding interactions of DHPS with active site residues. C, Mechanism of SLA reductases proposed based on YihU•NADH•DHPS crystal structure showing hydride transfer from C4 of the *si*-face of the nicotinamide ring of NADH to the carbonyl of SLA, with lysine-171 as general acid residue.

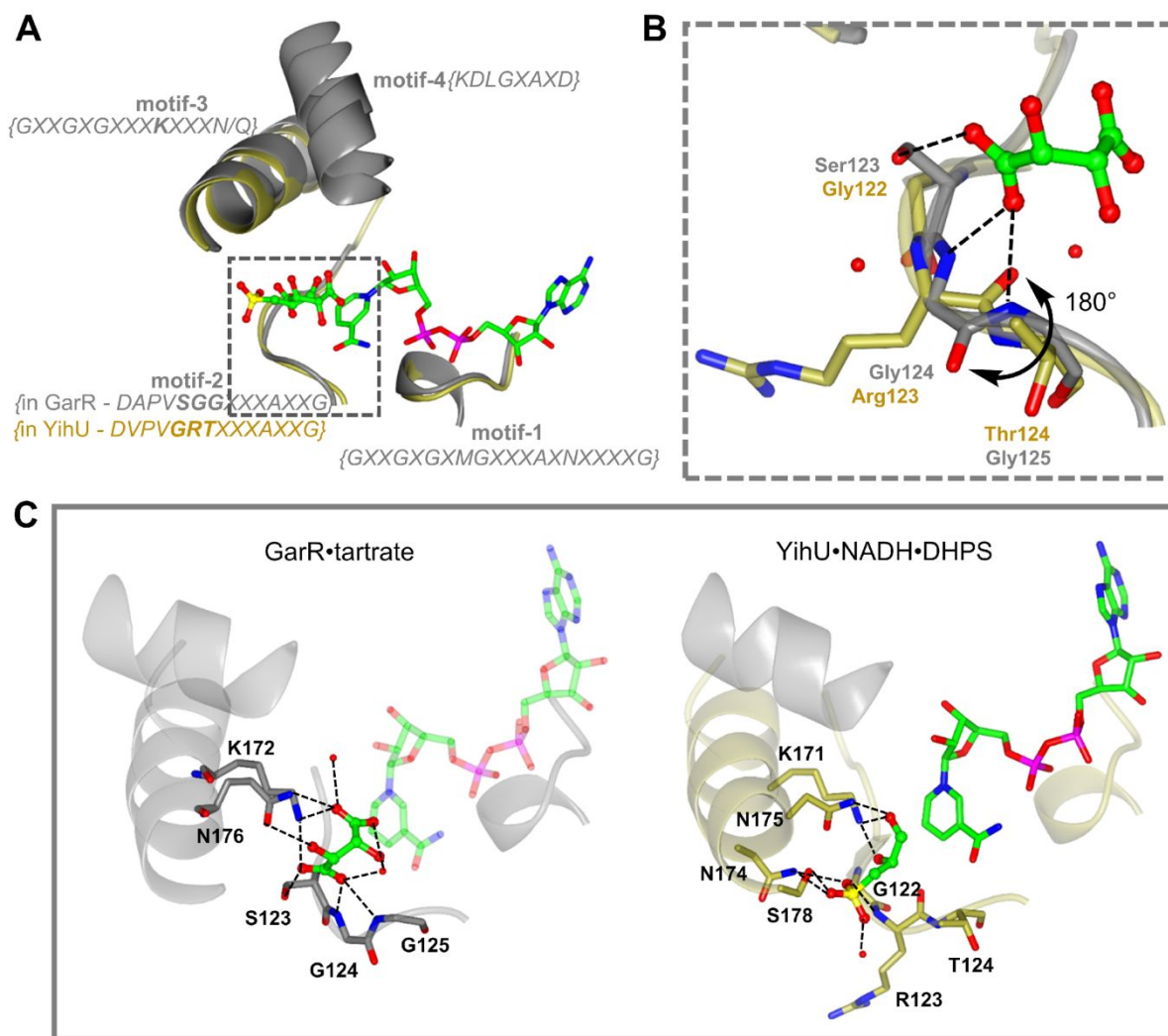


Figure 7. Flexibility in structurally conserved β -HAD motifs provide distinct binding modes for carboxylate versus sulfonate substrates. *A*, Overlay of motifs 1-4 in the YihU•NADH•DHPS dimer pair (in gold and grey) and GarR•tartrate monomer (in grey). *B*, Close-up view of the substrate binding loop containing *DAPVSSGG* in GarR and *DVPVGRT* sequence in YihU showing flip in backbone amides to bind the carboxylate substrate analog, L-tartrate. *C*, Comparison of carboxylate binding site in GarR•tartrate (1VPD) and sulfonate binding site in the YihU•NADH•DHPS structure. The NADH molecule is superposed here from the YihU•NADH•DHPS structure to illustrate the relative positioning of the cofactor in the GarR•tartrate structure.

Table 1. Kinetic parameters for YihU wildtype and mutants of residues in the sulfonate binding site.

| Enzyme | Variable substrate | k_{cat} (s^{-1}) | $K_{\text{M}}^{\text{app}}$ (mM) | $k_{\text{cat}}/K_{\text{M}}^{\text{app}}$ ($\text{mM}^{-1} \text{s}^{-1}$) |
|---------------|--------------------|--------------------------------------|----------------------------------|---|
| YihU wildtype | SLA ^a | 332 ± 9 | 0.30 ± 0.038 | 1090 ± 120 |
| | NADH ^b | 548 ± 28 | 0.082 ± 0.011 | 6720 ± 580 |
| YihU-G122S | SLA ^a | 71 ± 2 | 1.63 ± 0.11 | 43 ± 4 |
| | NADH ^b | 178 ± 9 | 0.123 ± 0.013 | 1450 ± 230 |
| YihU-R123G | SLA ^a | 68 ± 6 | 7.99 ± 1.02 | 8.5 ± 1.8 |
| | NADH ^b | 22.1 ± 0.5 | 0.009 ± 0.001 | 2360 ± 300 |
| YihU-T124G | SLA ^a | 27 ± 2 | 5.74 ± 0.79 | 4.7 ± 1.0 |
| | NADH ^b | 12.7 ± 0.6 | 0.023 ± 0.004 | 570 ± 130 |

^a [SLA] was varied while [NADH] was held constant at 0.1 mM.

^b [NADH] was varied while [SLA] was held constant at 5 mM.